IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re patent application of: BRENTANO *et al.*	)	Group Art Unit: 1634
Application Serial No. 10/665,708	)	Examiner: Switzer, J.C.
Filed: September 18, 2003	)	Atty. Docket No. GP107-03.DV1
For: NUCLEIC ACID AMPLIFICATION AND DETECTION OF *MYCOBACTERIUM* SPECIES	)	Confirmation No. 6892

DECLARATION OF STEVEN T. BRENTANO
UNDER RULE 132

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Commissioner for Patents
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Sir:

This Declaration accompanies and supports Applicants' response to the Office communication dated January 18, 2007 for the above application on which I am a named inventor.

I, Steven T. Brentano, Ph.D., declare and state as follows:

1. I am currently Associate Director of Technology Research and Bioinformatics at Gen-Probe Incorporated located in San Diego, California. I received a Bachelor of Science in 1981 from the University of California, San Diego with a major in Chemistry. I received a Ph.D. in 1987 from the University of Iowa. I was a postdoctoral researcher at the University of California, San Francisco from 1987 to 1991. From 1987 to the present I have worked continuously in the field of molecular biology. I am a member of the American Society for Microbiology (ASM),

American Society for the Advancement of Science (AAAS), and periodically am a peer reviewer of submissions for publication in various scientific journals. I am the author of twelve papers in the field of gene rearrangement, gene regulation and molecular diagnostics in national and international publications. I am an inventor named on fifteen issued U.S. patents and this U.S. patent application.

2. I have been employed by Gen-Probe Incorporated as a scientist since 1991 where I have performed research in the field of nucleic acid amplification, probe-based detection of nucleic acids, bioinformatics, and development of molecular diagnostic assays. During that time, I developed new methods and compositions for direct detection of nucleic acids and detection of amplified nucleic acids of pathogenic bacteria and viruses, as exemplified by U.S. patent numbers 7,094,542, 7,094,541, 6,946,254, 6,747,141, 6,664,081, 6,582,920, and 6,218,107. Also, during that time, I have developed improved methods and compositions for nucleic acid hybridization, amplification, and detection as exemplified by U.S. patent numbers 7,122,316, 7,070,925, 6,903,206, 6,602,668, and 6,534,273.

3. I assigned my rights in the present invention to Gen-Probe Incorporated and BioMerieux S.A. by an assignment of U.S. Serial number 60/172,190, recorded at the U.S. Patent and Trademark Office at Reel/Frame 010667/0788 on March 7, 2000. On information and belief, Gen-Probe Incorporated and BioMerieux S.A. now have rights in this invention.

4. I have read and understand the "Claim Rejections - 35 USC § 103" section of the Office action dated 01/18/2007 for this application. That section discusses the "Buck" reference which refers to the publication by Buck et al., "Design Strategies and Performance of Custom DNA Sequencing Primers" published in *BioTechniques*, vol. 27, no. 3, pages 528-536 (1999). I am familiar with the Buck et al. reference and provide the following comments on that reference and

a "factual showing" related to the conclusions drawn from that reference.

5. I have also read the comments related to the Buck et al. reference that were presented in an amendment for this application filed October 30, 2006, at pages 14 to 16. I agree with the comments related to the Buck et al. reference that appeared in that amendment. My comments refer to some of the same passages cited in the amendment related to the Buck et al. reference.

6. The Office action of 01/18/2007 (at page 7, lines 4-5) referred to Buck et al. as demonstrating "the equivalence of primers", which lead to the conclusion that every primer would reasonably be expected to function equivalently. I strongly disagree with this conclusion because the statements made by Buck et al. must be viewed as limited to the particular *in vitro* system that they used in the experiments. Also, I strongly disagree with this conclusion based on my personal experience in designing and testing primers in a variety of different *in vitro* systems.

7. Buck et al. describe a survey of primers used in DNA sequencing reactions (e.g., see page 529, col. 2, to page 530, col. 3 "The Survey" through "DNA Sequence Analysis"). To conduct this survey, Buck et al. selected an optimal test sequence that had been "previously shown to contain no segments that affect sequence ladder extension." Buck et al. also state that the plasmid used to make the test template "was preselected to contain a test sequence lacking obstacles to sequence extension" (see page 530, column 2, "Test Template and Preparation"). Clearly, Buck et al. limited their analysis to a single test sequence that was considered optimal and then used that test sequence in a single form of primer extension, i.e., DNA sequencing reactions. Therefore, even if Buck et al. considered their primers to be equivalent, the primers should only be considered to be comparable in this particular system, i.e., using this particular template in DNA sequencing reactions. Further, normally one skilled in the art will understand that many natural targets for organisms of interest most certainly do not consist of sequences

with those idealized features. Due to many different constraints, there is often little choice about where oligonucleotides must be designed for a useful in *vitro assay*. The sequences one must work with are dictated by nature and the function of the gene, not the choice of the investigator as was the case in Buck et al.

8. Throughout the paper, Buck et al. consistently, specifically and explicitly state that the results being reported are relevant to sequencing reactions using the template and reaction components specified. The authors make comparisons between the design guidelines used by those contributing primer sequences for both sequencing reactions and PCR, and discuss equivalence of primers for sequencing their idealized template, but never make any claims about equivalence of PCR primers. In fact, in the DISCUSSION section (p. 533, col. 1) they state "In this study, we examined the perceptions and strategies of participant core facility personnel for the design and selection of DNA sequencing and PCR primers. The results may reveal some unexpected misconceptions in the requirements of good sequencing primer design" (emphasis added). In other words, Buck et al. are consistent not to draw any conclusions about PCR primer performance throughout the paper.

9. For analysis of primer performance, Buck et al. used cycle sequencing with the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit from PE Biosystems. Sequencing products were analyzed on an ABI PRISM 377 DNA Sequencer. The output of such a sequencer, called the electropherogram, is analyzed automatically by the either the included ABI software, or by software from a third party. Buck et al. states that "the data" were directly exported to Sequencher software, presumably to interpret the electropherograms. In either case, the automatic functions of the software are programmed to make a best estimate of the predominant sequence derived from the electropherogram based on parameters including fluoresence peak height in each channel, peak spacing, and the presence of unidentified or

conflicting peaks. The software is not designed to flag or otherwise note ambiguities below a certain threshold. It is well known by those with normal skill in the art that contaminating peaks representing sequences present at about 5-10% or less are effectively ignored by the software. Buck et al. make reference to the contributions of the reagent kit used in decreasing background peaks, and the effect this has on improving sequence readout: "thus background peaks are less likely to interfere with base calling, lessening the overall effects of potential problems like secondary priming and stutter" (p. 536 col. 1). Thus, while Buck et al. report very high quality results from most primers, it must be understood that any low-level contaminating sequences may be minimized by choice of reagents, and are automatically rejected by the software. Therefore, while the study is well designed to determine if the primers are effective for sequencing in this context, the sequencing method itself is an insensitive tool for determining if mispriming events are occurring.

10. I also noted that Buck et al. used methods to purify the template that molecular biologists consider to be labor intensive and time consuming, but that produce extremely pure samples. See page 535, col. 2: "the plasmid template was selected for absence of sequence extension obstacles and purified by double banding in CsCl-ethidium bromide isopycnic density gradients. Therefore, this template was extremely pure and optimal for sequencing." Such extraordinary efforts made extremely pure samples for testing for Buck et al.'s survey, but that is not the norm for samples used in most primer-based reactions. For example, most laboratories do not use CsCl-ethidium bromide isopycnic density gradients but instead use a less demanding purification process to prepare a template for primer-based reactions, such as the process described in U.S. Patent 6,534,273, of which I am a coinventor.

11. An additional factor in the procedure used by Buck et al. is that besides being highly purified, only a single sequence was present in the reaction. It is rarely the case that an *in vitro*

assay will be used on a single highly purified single target nucleic acid composed of a nucleotide sequence of the users choosing. One with normal skill in the art of designing oligonucleotides for *in vitro* assays will certainly understand that actual samples almost always contain a vast variety of contaminating nucleic acids.

12. An additional factor in the procedure used by Buck et al. is that besides being highly purified, only a single target sequence was present in the reaction. It is quite commonly the case that an organism targeted in an *in vitro* assay will actually consist of many different genetic sequences which all must be amplified and detected. As one example, there are hundreds of genetic variants of HIV described in the literature, and an assay that would detect "HIV" must detect all the variants to be useful. This complication is not considered at all in the simplistic target model presented by Buck et al. Oligonucleotide sets that detect "HIV" for example, must undergo a much more rigorous selection process to be useful.

13. Further, actual samples used in *in vitro* assays will most likely contain non-target nucleic acids that must not be detected to avoid a false positive signal. As an example, one may wish to detect *Mycobacterium tuberculosis* rRNA, but not *M. avium* rRNA (which have very similar rRNA sequences) or other bacterial nucleic acids which may be present in the same sample. Simply having the sequence of *M. tuberculosis* will not allow one to design a successful assay that discriminates *M. tuberculosis* from *M. avium*. This is another case in which the oligonucleotide selection process is much more involved than the simplistic set of circumstances presented by Buck et al.

14. Buck et al. only consider a reaction composition in which only a single oligonucleotide is present in the reaction. One with normal skill in the art will know that amplified reactions almost always have more than a single oligonucleotide present in the reaction. The presence of

two or more oligonucleotides significantly complicates the primer selection process: to name a few, designs must minimize cross priming, minimize primer interactions, and maximize target selectivity and inclusivity as stated above. These critical parameters were certainly not considered by Buck et al. in their analysis.

15. Each of the participants in the Buck et al. survey designed an unknown number of primers but only submitted their "best primers" for testing. The submitted primers were tested along with 95 control primers made from sequential sequences at 3-bp intervals on the test sequence (see page 530, col. 1, "Control and Submitted Primers"). The primers were synthesized, purified, and tested individually in DNA sequencing reactions by using the highly purified template under specific conditions (see page 530, col. 2-3, "DNA Sequence Analysis"). Based on this information about how Buck et al. conducted their tests, my opinion is that any conclusions that Buck et al. provide based on this survey must be limited to the system used to conduct the survey and cannot be relied on as "evidence of the equivalence of primers" in any other test system.

16. Buck et al. also reported that varying only <u>one</u> condition in their test system changed the performance of the primers (see page 535, col. 1, "Old Rhodamines vs. dRhodamines"): "The old rhodamine dye reactions were more strongly impacted by primer physico-chemical characteristics than the dRhodamine dye reactions." In other words, changing <u>one</u> reagent in the test system (using old rhodamine or dRhodamine) changed the results. Buck et al. stated that "the former reactions [old rhodamine] seemed more sensitive to primer characteristics." Clearly, Buck et al. recognized that the primers did *not* all perform equivalently, and even one change in the reaction conditions revealed differences in the "primer characteristics." Thus, Buck et al. included statements that show the primers had different characteristics and that the reaction conditions or reagents revealed those characteristics. These statements in Buck et al. reveal that the primers did not all perform equivalently.

17. I strongly disagree with the conclusion that Buck et al. provide "evidence of the equivalence of primers" for all of the reasons discussed above and it is my opinion that any conclusions based on Buck et al.'s survey must be limited to their particular test and analysis system. To expand the conclusions of Buck et al. to the "equivalence of primers" in any *in vitro* system for any target sequence is unsupported.

18. An expansion of the conclusions of Buck et al. to "the equivalence of primers" in all test systems ignores other information that was known to molecular biologists at the time the invention of this application was made. For example, it was known that some sequences contain structure that interferes with primer extension, which Buck et al. acknowledged when they chose a template sequence "selected for absence of sequence extension obstacles." Such structure is generally referred to as secondary or tertiary structure, such as described in US Patent 5,677,128 (Hogan et al., 1997) which was cited in the Office action dated July 28, 2006 for this application: "Biological and structural constraints on the rRNA molecule which maintain homologous primary, secondary and tertiary structure throughout evolution, and the application of such constraints to probe diagnostics is the subject of ongoing study." (See col. 6, lines 40-44.) US Patent 5,677,128 also cited many scientific references that demonstrate the wide spread knowledge of secondary structure in nucleic acids in the "Other Publications" section:

Carbon et al., "The sequence of the 16S RNA from Proteins vulgaris. Sequence comparison with E. coli 165 RNA and its use in secondary structure model building," Nucleic Acids Research 9:2325-2333 (1981);

Fox and Woese, "55 rRNA secondary structure," Nature 256:505-507 (1975);

Hori and Osawa, "Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 54 5S RNA species," Proc. Natl. Acad. Sci. USA 76:381-385 (1979);

Luehrsen and Fox, "Secondary structure of eukaryotic cytoplasmic 5S ribosomal RNA," Proc. Natl. Acad. Sci. USA 78:2150-2154 (1981);

Stiegler et al., "A General Secondary-Structure Model for Procaryotic and Eucaryotic RNAs of the Small Ribosomal Subunits," Eur. J. Biochem. 120:484-492 (1981);

Veldman et al., "The primary and secondary structure of yeast 26S rRNA," Nucleic Acids Research 9:6935-6953 (1981); and

Woese et al., "Secondary structure model for bacterial 16S ribosomal RNA: phylogenetic, enzymatic and chemical evidence," Nucleic Acids Research 8:2275-2293 (1980).

Thus, it was well known to skilled molecular biologists that not all sequences that are potential targets for primer-based assays are the same, and some of them are known to contain secondary structure that can interfere with primer extension.

19. The following remarks present factual information to show that all primers to a known *Mycobacterium* sequence do not function as equivalents. This information is based on experiments that were performed and recorded contemporaneously with the research that lead to the invention of this application. The experiments that I summarize here directly compared the relative efficiencies of primer oligonucleotides in *in vitro* nucleic acid amplification reactions similar to those disclosed in this application, but using different promoter primers that differed from each other by only a single nucleotide. That is, the same target sequence (20 copies per reaction of *M. tuberculosis* rRNA) was tested under the same conditions by using a variety of different promoter primers (variable primers) in combination with a single second primer oligonucleotide (a constant primer). For each combination of primers (the variable promoter primer and constant primer), fifteen reactions were performed. For each set of tests, the amplified nucleic acids were detected under the same conditions by using the same labeled oligonucleotide probe to provide a detectable signal ("relative light units" or "RLU") that measures of the efficiency of amplification, by using methods similar to those described in this application. The results of these tests are reported here as the average (mean) RLU obtained for the primer combinations for each of the variable promoter primers and the percentage of failures

(% failures) of amplification among the 15 reactions performed for each primer combination. "Failures" refers to reactions in which the detected signal fell below a predetermined cut off quantity to indicate that the reaction did not produce sufficient amplified nucleic acid to be considered a positive result. The variable primers are described using a Greek letter (e.g, α, β, γ) to represent the sequence that was identical between a group of variable primers, followed by a letter (A, T, C, or G) to indicate the different single bases in the same nucleotide location between the variable primers in that group of primers. For example, a group of variable primers is made up of α-A, α-T, α-C and α-G have the same sequence except for a single locus in which the base A, T, C or G was used in the individual different members of the α group. The results shown below demonstrate that primers that vary from each other by as little as a single nucleotide base in the oligonucleotide perform differently under the same conditions. For example, in Experiment 1 the relative efficiencies in terms of amplified product (average RLU) showed variable primer α-C to be the best primer which resulted in about 10-fold or more amplified product compared to the other variable primers in the group (α-A, α-G, or α-T), which showed high percentages of amplification failures in the 15 reactions. In Experiment 2, β-C performed amplification better than β-T because β-C had no amplification failures, but the average RLU detected for the 15 reactions that used the β-C primer was somewhat less than observed for the 15 reactions that used the β-T primer.

Experiment	Variable Primer	Average RLU	% CV	% Failure
1	α-C	585,418	102	0
	α-G	36,517	220	60
	α-A	60,448	187	67
	α-T	39,390	125	60
2	β-C	890,207	46	0
	β-T	1,048,782	130	33

In a third set of experiments, the variable primers differed from each other at two nucleotide positions in the oligonucleotide. Using the same format as described above, these variable primers are shown with a Greek letter and the base letters of the two variable bases (γ-CC, γ-GA, γ-AA, and γ-TA). These variable primers were tested in amplification and detection reactions as described above for Experiments 1 and 2, using 20 copies of the same target sequence. Based on both the differences in average RLU detected and particularly the % failures of amplification among the 15 reactions performed for each of the combinations that included the variable primers of the γ-CC, γ-GA, γ-AA, and γ-TA group, the data shows that the primers performed differently, with primer γ-CC having the lowest percentage of failures.

Experiment	Variable Primer	Average RLU	% CV	% Failure
3	γ-CC	208,322	106	33
	γ-GA	126,101	200	73
	γ-AA	309,245	357	87
	γ-TA	334,914	270	80

The results of these experiments shown above provide evidence that not all primers perform equivalently.

20. Based on the comments above, it is my opinion that not all primers act equivalently. The combinations of primers that are disclosed and claimed in this application represent selected combinations that performed in vitro nucleic acid amplification of *Mycobacterium* 16S rRNA or a DNA encoding 16S rRNA in a manner that provided superior results. These combinations of primers produced quantities of amplified nucleic acids of a quality that allowed them to be detected in a variety of ways, e.g., by using a labeled oligonucleotide probe or by labeling the

amplified nucleic acids, hybridizing them to an immobilized array of DNA probes, and detecting a pattern of fluorescent signals on the array. For example, Table 4 in the specification shows that the claimed primers of SEQ ID numbers 21-24 efficiently amplified *Mycobacterium* 16S rRNA sequences from *Mycobacterium* species (*M. xenopi* and *M. celatum*).

21. Despite the recognized importance of nucleic acid amplification methods used for detection of pathogenic bacteria, there remains a need for a method of amplification that efficiently amplifies different species of *Mycobacterium* by using a limited number of primers to produce amplified products of sufficient quantity and quality to allow detection of the *Mycobacterium* species present in a biological sample. Based on the results of experiments done in the research leading up to filing of this patent application, it is my opinion that the primers disclosed and claimed in this application fulfil that need.

22. Based on the remarks provided herein with regard to the Buck et al. reference, it is my opinion that the reference does not provide evidence of the equivalence of primers in all primer-based *in vitro* reactions. Based on my personal experience related to primer designs and testing in amplification reactions, including those that lead to the inventions claimed in this application, it is my opinion that the inventions claimed in this application are not made obvious by the combination of references cited in the Office action that included the Buck et al. reference.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S. Code § 1001, and that such willful, false statements may jeopardize the validity of the application or any patent issuing therefrom.

Respectfully submitted,

Dated: 3-19-07

By: Steven T. Brentano

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